Anaerobic ammonium oxidation (anammox) with planktonic cells in a redox-stable semi-continuous stirred-tank reactor

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Running title: Planktonic anammox cultivation in semi-CSTR

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Abstract

Anaerobic ammonium oxidizing (anammox) bacteria are routinely cultivated in mixed culture in biomass-retaining bioreactors or as planktonic cells in membrane bioreactors. Here we demonstrate that anammox bacteria can also be cultivated as planktonic cells in a semi-continuous stirred-tank reactor (semi-CSTR) with a specific growth rate μ of 0.33 d^{-1} at 30°C. Redox potential inside the reactor stabilized at around 10 mV (±15 mV) (vs standard hydrogen electrode) without gas purging. Reactor headspace pressure was used as a sensitive and real-time indicator for nitrogen evolution and anammox activity. The reactor was dominated by an organism closely related to "*Candidatus* Kuenenia stuttgartiensis" (~87% abundance) as shown by Illumina amplicon sequencing and fluorescence *in situ* hybridization. Epifluorescence microscopy demonstrated that all cells were in their planktonic form. Mass balance analysis revealed a nitrite/ammonium ratio of 1.270, a nitrate/ammonium ratio of 0.238, and a biomass yield of 1.97 g volatile suspended solids per mole of consumed ammonium. Batch experiments with the reactor effluent showed that anammox activities were sensitive to sulfide (IC_{50} = 5 μM) and chloramphenicol (IC_{50} = 19 mg L^{-1}), much lower than reported for granular anammox biomass. This study shows that semi-CSTR is a powerful tool to study anammox bacteria.

Introduction

Anaerobic ammonium oxidation ("anammox") is a microbially catalyzed process of great importance both in the natural nitrogen cycle and in technical applications for removal of fixed nitrogen. In this process bacteria gain energy by oxidizing ammonium, channeling the electrons through an energy-fixing electron transport chain and reducing nitrite to nitrogen gas. The overall reaction is driven by a redox potential difference of about 400 mV (under standard conditions). In comparison with standard nitrogen removal, anammox plants can save energy and co-substrates. To enable anammox, both ammonium and nitrite must be present at the same time. Such conditions occur in natural redox gradient, but in technical plants delicate control is necessary.

In spite of strong efforts, it has not yet been possible to obtain a pure bacterial anammox culture but detailed studies in mixed cultures revealed the phylogenetic affiliation,[1] the morphology,[2] physiology,[1,3] and biochemistry[4,5] of the catalyzing bacteria. Metagenomics approaches have revealed partial genomic information.[6-12] In the context of our study it is important to emphasize i) that all known anammox organisms cluster within the orders Brocadiales or Planctomycetales[13] of the phylum Planctomycetes,[14] ii) that all anammox organisms described grow slowly, with specific growth rates μ of 0.022 to 0.33 corresponding to doubling times t_d between 32 and 2.1 days,[13,15] and iii) that anammox cells contain an anammoxosome, a DNA-free compartment containing all directly-involved enzymes.[14,16]

One of the first described and best characterized anammox organisms is "*Candidatus* Kuenenia stuttgartiensis", which is a physiological generalist, also able to use iron and manganese as electron acceptors and a wide variety of electron donors for respiration.[11] Growth rates are described to be 0.06-0.23 (doubling times of 3-11 days).[17,18] Its first draft genome was sequenced in 2006,[11] and first proteomic analyses with a focus on anammoxosome proteins were described.[19] Genes for the biosynthesis of flagella were found suggesting that it could express a flagellum enabling the bacteria to freely swim e.g. within oxygen gradients.[14]

Due to the long doubling times, most research groups cultivated anammox bacteria in biomass-retaining reactors such as upflow anaerobic sludge blanket, sequencing batch, or membrane bioreactors to achieve high cell densities and high volumetric anammox reactivity.[17,20,21] However, the formation of sludge or granules can strongly influence physiological and biochemical characteristics and only in membrane bioreactors was it possible to grow anammox cells in planktonic form.[17]

The goal of the current study was to establish active anammox bacteria in a simple continuous stirred-tank reactor. By eliminating biomass retention we sacrificed high cell density in favor of culture homogeneity, sub-cultivation reproducibility, long-term reactor stability and high growth rates. We hypothesized that such a system is suitable to characterize physiological properties of the inhabiting microorganisms and to optimize cultivation conditions in the lab. In addition, we hypothesized that we can use the effluent as a stable and homogeneous source for batch cultivations, enrichments and eventually isolation of anammox organisms. Within such batch cultivations we aimed at the characterization of the inhibitory effects of various effectors such as salt, nitrite, sulfide, and phosphate to compare IC_{50} values between free-living and granular anammox biomass. Chloramphenicol, a potential inhibitor of denitrifying microbial populations and denitrification enzymatic activity,[22] and methylene blue, a redox indicator potentially useful for anammox cultivation, were specifically evaluated in this study.

To achieve these goals, we here describe a semi-continuous stirred-tank reactor that is tightly controlled especially in regard to redox-conditions, but also for pH, temperature, nutrients, electron donors and electron acceptors and in which we use the headspace pressure as a key parameter for reactor control. The reactor runs reproducibly with "*Ca.* K. stuttgartiensis" in planktonic form at high growth rates. Batch cultivations with the effluent confirmed the success of the approach. Although the main goal of this study was the description of reactor parameters at lab scale, our approach will be extended to the description of reactors at pilot or full scale in the future.

Material and Methods

Bacterial inoculum and media

Anammox sludge granules from a sequencing batch reactor treating landfill leachate were kindly provided by Prof. Yongzhen Peng (Beijing University of Technology) and were handled in an anaerobic glovebox to provide strictly anoxic conditions.

Both, initial batch cultivation and reactor cultivation were done in an anoxic defined synthetic mineral medium containing vitamins but no reducing agents. For initial batch cultivation in serum bottles, cultures were inoculated with 10% vol/vol. To inoculate from granules, bottles were opened inside an anaerobic glovebox and the inoculum was transferred with a sterile spatula. The reactor was semi-continuously fed from three different solutions with ammonium, nitrite and mineral salts as the principal components. All feeding solutions were degassed and purged with nitrogen to remove oxygen. Details are described in the Supporting Information (SI) Methods.

Reactor components and operation

The reactor vessel was a "Widdelkolben"[23], an inverted 2.4 L Erlenmeyer flask with ports for medium amendments, sampling and gas pressure measurements which were all done automatically and controlled via a Raspberry Pi microprocessor (Figure 1 and SI Methods).

The reactor contained 2 L culture liquid and 400 mL headspace. Periodically, every 43-100 minutes, depending on the chosen hydraulic retention time, 20 mL reactor suspension was pumped from the reactor into the effluent bottle, and was replaced with 20 mL fresh medium (10 mL 40 mM nitrite medium, 5 mL 120 mM ammonium medium and 5 mL salt medium). Details are given in the SI Methods. Ammonium was always in excess to maintain a low redox potential and non-toxic nitrite concentrations. The buildup of headspace pressure was continuously monitored and when it reached 7.71 kPa (sensor voltage output of 500 mV), a gas valve was opened for 30 s to release gas into the gas collection bottle. This low threshold was set to minimize disturbance of the CO_2 layer in the reactor headspace.

Incubation of reactor effluent with inhibitory compounds

In an anaerobic glovebox, 60 mL nitrogen-flushed glass bottles were filled with 50 mL reactor effluent and closed with butyl rubber septa and aluminum crimp caps. Effectors were added within an anaerobic glovebox with sterile syringes through the septa or directly added to the bottles as powder. Except for the experiment in which nitrite was varied, 3 mM nitrite was added at time 0. Each bottle was directly connected to a MPX5100DP pressure sensor via a 0.41 mm Luer Lock needle. All sensors were electrically connected to a LabJack converter for data reading and storage. Pressure sensors were read every 3 seconds. In total, twelve such bottles were set up for each experiment, allowing the simultaneous testing of five different effector concentrations in duplicate plus two bottles as negative controls without effector. Consistency of the obtained data between replicate bottles and correlation of pressure build-up and effector concentration was demonstrated in an experiment where we tested the effect of chloramphenicol on anammox activity (SI, Figure S1). In total, six effectors were tested in this study: salt (NaCl, 30-940 mM), nitrite (3-35 mM), sulfide (Na_2S, 0-500 μM), phosphate (mixture of K_2HPO_4 and KH_2PO_4, pH 7.2, 1.5-51.5 mM), chloramphenicol (0-1 g L^{-1}), and methylene blue (0-10 mg L^{-1}).

Experiments were incubated for 24 hours after which 3 mL air was injected into each bottle for calibration of the pressure sensor readings. Gas production rates were calculated as mL per day across adjacent 1,000 consecutive data points (3,000 seconds). Maximum gas production was typically reached after 10-14 hours. The same time frame was chosen for all twelve bottles in one experiment to calculate anammox activities from the slopes.

Analytical and microscopic techniques

Ammonium concentrations were determined with a modified salicylate method.[24] In brief, samples were diluted to an ammonium concentration below 0.15 mM, and 200 μL of these diluted samples were sequentially mixed in a 96-well microplate with 50 μL bleach solution (120 mM NaOH, 0.15% w/v sodium hypochlorite) and 50 μL catalyst solution (120 mM NaOH, 10% w/v sodium salicylate, 0.04% w/v sodium nitroferricyanide). Absorbance at 650 nm was measured on a Synergy 2 microplate

reader (Biotek, USA). This ammonium quantification method typically yielded a standard curve linearity of >0.999 and a low standard deviation (<5%) among triplicates. Nitrate concentrations were determined on a Dionex DS-120 ion chromatography with an IonPac AS4A-SC column and an eluent containing 0.7 mM $NaCO_3$ and 0.7 mM $NaHCO_3$ at a flow rate of 1 mL min^{-1}. Due to tailing of the chloride peak that immediately preceded the nitrite peak, nitrite could not be reliably quantified by ion chromatography. Therefore, nitrite concentrations were determined separately with the Griess reagent kit (Molecular Probes) according to the manufacturer's instructions. The detection limit of nitrite was at ~0.05 mM.

To measure volatile suspended solids (VSS), 200 mL reactor effluent was filtered through 47-mm diameter Whatman GF/F glass fiber filter (pore size 0.7 μm, GE Healthcare, USA) previously baked at 550°C for 1.5 h. After filtration the filter was dried overnight at 105°C and weighed to obtain the total suspended solid (TSS) value. Then the filter was combusted at 550°C for 1.5 h to obtain the VSS value from the difference of the weight. VSS analysis was done in triplicate.

Direct epifluorescence microscopic counting of SYBR-green stained cells on agarose-coated slides and fluorescence *in situ* hybridization (FISH) of cells with 16S-rRNA-targeting probes are described in the SI Methods.

Microbial community analyses

One milliliter of culture liquid was centrifuged at 10,000 g for 15 min at 4°C, and the resulting pellet was used for extraction of genomic DNA using DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions, except that 20 μL of lysozyme (100 mg mL^{-1}) were added to facilitate cell lysis. Extracted DNA samples were stored at -20°C and used as template for clone library construction and Illumina sequencing.

PCR amplification of 16S rRNA genes for clone library construction was done for 30 cycles using the forward primer (338F 5'-ACT CCT ACG GGA GGC AGC AG-3' and 338F-AMX 5'-ACT CCT ACG GGA GGC TGC AG-3', 0.05 μM each) and the reverse primer 1392R 5'-ACG GGC GGT GTG TRC-3' (0.1 μM) with the annealing temperature of 55°C. Purified PCR products were ligated onto pGEM-

T vector (Promega) which was later transformed into *E. coli* competent cells. Clones were sequenced by Sanger sequencing using the primers provided with the vector. Sequences have been deposited in GenBank database under accession numbers MH040190 to MH040197.

For Illumina amplicon sequencing of the DNA from the initial batch culture, V3-V4 region of 16S rRNA genes were amplified using the forward primer 338F 5'-ACT CCT ACG GGA GGC AGC AG-3' and the reverse primer 806R 5'-GGA CTA CHV GGG TWT CTA AT-3'. Illumina MiSeq sequencing (300 bp paired-end reads) was performed by BGI (Shenzhen, China). For Illumina amplicon sequencing of the DNA from the reactor effluent, V3-V4 region of the 16S rRNA gene were amplified using the forward primer 341F 5'-CCT ACG GGN GGC WGC AG-3' and the reverse primer 785R 5'-GAC TAC HVG GGT ATC TAA TCC-3'.[25] Illumina MiSeq sequencing (300 bp paired-end reads) was performed by LGC Genomics (Berlin, Germany). All sequence reads were processed using the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3).[26] OTU clustering threshold was 0.98 sequence identity. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 128). Classification threshold was 0.93 sequence similarity. Visualization of microbial population abundance was done with Krona (www.krona.sourceforge.net).[27]

The amplicon sequencing reads have been deposited under NCBI BioProject PRJNA437226, with accession numbers SRX3778537 and SRX3778536 for the batch culture and the reactor effluent, respectively.

Results

Batch cultivation and microbial community analysis

Suspended granules (30 mL) were obtained in August 2013 from an anammox reactor and stored under nitrogen gas in a sealed 50-mL centrifuge tube at 4°C for almost one year because no enrichment was planned at the time of sample receipt. The anammox enrichment process was started in July 2014 using the stored sample, and 5 mL of the granules were transferred to 90 mL of batch

cultivation medium containing 3 mM nitrite and 4.6 mM ammonium. After two months of incubation, nitrite was depleted in the bottle, and a 10% v/v inoculum was transferred to fresh medium. After another two such transfers we obtained an active anammox culture free from granules and sludge. The culture catalyzed conversion of 3 mM nitrite in ten days with concurrent decrease of ammonium concentrations (Figure 2). A population analysis by sequencing clones from a 16S rRNA gene clone library indicated that about a quarter of the detected 16S rRNA gene sequences (26.7%) were affiliated with "*Ca.* Kuenenia stuttgartiensis", whereas 56.7% were affiliated with the genus *Pseudomonas* (SI, Table S1). With the same DNA sample, an Illumina amplicon sequencing was performed, confirming that the culture was dominated by organisms described to catalyze the anammox process ("*Ca.* Kuenenia stuttgartiensis", 17.2% of the detected 16S rRNA gene sequences) and *Pseudomonas* (70.3%), while the other OTUs were all at lower shares than 2% (Figure 3A).

To obtain sufficient culture liquid to inoculate the reactor, 100 mL of granule-free culture liquid was transferred to 900 mL of medium containing 3 mM nitrite via nitrogen-flushed syringes and needles. After 15 days of incubation the nitrite concentration was below the detection limit and the 1-L culture was used for reactor inoculation.

Reactor cultivation of "*Ca.* Kuenenia stuttgartiensis"

A gas-tight semi-continuous stirred tank reactor (semi-CSTR) was constructed allowing strictly anoxic reactor operation and real-time monitoring of pressure, redox potential, pH and temperature (Figure 1 and SI Methods). It allowed the amendment of nitrite and ammonium as the two main energy-providing molecules from separate medium bottles. To start the reactor, the 1-L stem culture described above was mixed with 1 L fresh batch cultivation medium containing 3 mM nitrite in the anaerobic glovebox and transferred to the reactor. The reactor was started with a hydraulic retention time of 6.9 days, and influent nitrite and ammonium concentration of 20 mM and 30 mM, respectively. During operation, the concentration of nitrite in the reactor effluent was constantly close to zero and gas was produced continuously, demonstrating that the injected nitrogen compounds were used in the reactor. When we decreased the hydraulic retention time by stepwise increase of the frequency of 1% medium

replacement, the effluent nitrite concentration stayed below 0.05 mM. In this way, the hydraulic retention time was shortened to 3 days after 16 days of operation, corresponding to a dilution rate of 0.33 d^{-1} (SI, Figure S1). Note that in the well-mixed CSTR, the hydraulic retention time equals the solid retention time, and therefore the dilution rate is the reciprocal of the hydraulic retention time at steady state conditions. The chosen medium composition and reactor operation effected that the pH was constantly between 7.3 and 7.6.

Previous studies on anammox bacteria showed that anammox activity depended on the redox potential in the cultivation vessels,[28] and could be suppressed by nanomolar levels of oxygen.[29] We therefore planned to provide a reducing agent to control the redox potential. However, long-time operation of the reactor for over one year demonstrated that with the described reactor design, degassing of feeding solutions and operation under exclusion of oxygen, no redox adjustment was necessary to maintain redox potential inside the reactor, which stayed at around 10 mV (± 15 mV) vs the standard hydrogen electrode (SHE). When the reactor flow was halted, the redox potential rose gradually to ~30 mV within 26 h (SI, Figure S2). Once the reactor flow resumed, the redox potential instantly stopped rising and slowly returned to the original level. After interrupting the reactor flow it took about 10 h for gas production to completely cease, and another 5 h to regain full gas production rate after resuming the reactor flow.

Reactor recovery

The reactor effluent was collected in nitrogen-flushed 2-L bottles and stored in the dark at 4°C to be used later for inhibitor tests (see below) or as inoculum when the reactor collapsed. During one-year operation under experimental conditions, the reactor collapsed three times under three different critical conditions which were introduced unintentionally: 1) after the reactor was incubated for 24 h at 38.6°C without feeding nitrite, 2) after we added a pulse of 0.2 mM Na_2S, and 3) after we added a pulse of 65 µM $FeCl_2$ from a separate $FeCl_2$ stock solution. The first two conditions led to complete loss of anammox activity, while the third condition resulted in the formation of a thick precipitate in the reactor which was presumably ferric oxides[30] and consequently stopped reactor operation. After such

reactor collapses, the reactor could be reproducibly reestablished with the stored eluent bottles. The activity in the effluent bottles was recovered, as judged from pressure production rates, within only 2 days after a bottle was warmed up to room temperature, spiked with 3 mM nitrite and incubated at 30°C. Effluent could be stored at 4°C for at least three months without startup delay.

Microbial community composition during reactor operation

Under steady state conditions with a dilution rate of ~0.33 d^{-1} (hydraulic retention time of 3 days), no granules developed in the reactor and the liquid changed to an orange color as has been described for other anammox cultures.[31] We performed FISH and Illumina amplicon sequencing after three-month reactor operation at a dilution rate of ~0.33 d^{-1}. The microbial cell density within the reactor was 7.6 ± 1.3×10^{7} mL^{-1}. Direct microscopic analysis of SYBR Green stained cells also showed that the cells were not assembling in aggregates, indicating that all cells in the reactor were in a planktonic form (SI, Figure S3). The majority of the cells showed the typical morphology of anammox cells with a ring or "c"-type shape indicating the presence of a DNA-free anammoxosome.[19] The fluorescence intensity was influenced by the concentration of SYBR Green dye applied and the incubation temperature (SI, Figure S4).

To quantify the abundance of anammox-catalyzing organisms in the established reactor culture, FISH was conducted using FAM-labeled EUB338-mix and cy5-labeled AMX820 probes for Eubacteria and anammox bacteria, respectively, and DAPI as DNA-counterstain. Samples taken from the reactor running steadily at a dilution rate of ~0.33 d^{-1} showed that the abundance of anammox organisms increased to ~85% (Figure 4).

A second Illumina amplicon sequencing of the established reactor culture showed that the relative share of "*Ca*. Kuenenia" 16S rRNA genes of the total detected 16S rRNA genes had increased to around 75%, while bacteria of the genus *Pseudomonas* almost disappeared (0.007%) and were replaced by heterotrophs such as *Methyloversatilis* (10.6%), *Azoarcus* (2.8%), *Melioribacter* (3.4%), *Rubellimicrobium* (2.0%), and several genera within the *Lentimicrobiaceae* (1.5%) (Figure 3B). If the genome of the "*Ca*. Kuenenia stuttgartiensis" strain in the reactor contains only one 16S rRNA gene

copy as described for the draft genome assembly of "*Ca*. Kuenenia stuttgartiensis"[11] and the other detected strains in the reactor culture have the 16S rRNA gene copy numbers as given in *rrn*DB,[32] the adjusted anammox cell fraction in the reactor was around 87%.

Stoichiometry of anaerobic ammonium oxidation in the reactor

To calculate the stoichiometry of the anammox process, a correlation between the cell density and VSS was first obtained. On day 30, the reactor effluent had a cell density of 8.5×10^{7} mL^{-1} and a VSS value of 48.1 (±1.2) mg L^{-1}, giving a per cell weight of 5.65×10^{-13} g VSS which was used to convert cell density to VSS in the nitrogen mass balance experiments below.

During a two-week monitoring period with a hydraulic retention time of 5.6 days, all nitrogen-containing compounds flowing into and out of the reactor were measured (SI, Table S2) including ammonium and nitrite in the reactor influent, ammonium, nitrite, nitrate and biomass in the reactor effluent, and nitrogen gas evolution. The total cell density was converted to anammox cell density using the anammox abundance observed above (87%). The elemental composition of the biomass was assumed as $CH_2O_{0.5}N_{0.15}S_{0.05}$ according to published results for "*Ca.* Brocadia" [33]. During the monitoring period, 252.5 and 249.7 mmol of nitrogen flowed in and out of the reactor, respectively, representing an almost closed mass balance (99.64%).

Data reconciliation[34] of the above measurements balancing nitrogen in- and output (SI, Table S2) resulted in a balanced anammox stoichiometry as shown in Equation 1. Based on this stoichiometry, biomass yield was 1.97 g VSS per mole of consumed ammonium.

$$1\,NH_4^+ + 1.270\,NO_2^- + 0.077\,HCO_3^- + 0.108\,H^+ = 1.010\,N_2 + 0.238\,NO_3^- + 0.077\,CH_2O_{0.5}N_{0.15} + 2.016H_2O \quad \text{Equation 1}$$

Incubations of the reactor effluent with various effectors

An important advantage of the CSTR design was the homogeneity of the reactor effluent which can be used for physiological characterization of anammox bacteria. For that we incubated the reactor effluent in closed bottles and monitored the pressure build-up in the headspace over time (Figure 5).

For the six tested effectors, IC_{50} values were 183 mM (NaCl), 9.6 mM (nitrite), 5 μM (sulfide), >51.5 mM (phosphate), 19 mg L^{-1} (chloramphenicol), and 53 μg L^{-1} (methylene blue). No apparent inhibition was observed with salt concentrations up to 120 mM, while 270 mM caused 75% inhibition. Nitrite at 6 mM caused 24% inhibition. Sulfide severely inhibited anammox activity already at the very low concentration of 5 μM. Chloramphenicol induced 57% inhibition at a concentration of 30 mg L^{-1}. An increase of anammox activity by 16% was observed when the phosphate concentration was increased from 1.5 mM to 3.5 mM. At 6.5 mM phosphate, anammox activity was 11% higher than in the controls at 1.5 mM. Therefore, a higher phosphate concentration may enhance anammox activity also in the reactor. Still 65% of the anammox activity was preserved at the highest tested concentration of 51.5 mM phosphate. Whereas methylene blue caused no apparent inhibition of anammox activity at 10 μg L^{-1}, 100 μg L^{-1} methylene blue caused 86% inhibition.

Discussion

Semi-CSTR is a promising reactor design for anammox cultivation

In this study, a semi-CSTR was successfully used for the cultivation of anammox cells in their planktonic form. This and the previously described membrane bioreactors[17] are the current two reactor designs that produce planktonic anammox cells. Membrane bioreactors have been used to cultivate "*Ca.* Kuenenia stuttgartiensis" (μ = 0.23 d^{-1}), "*Ca.* Brocadia sapporoensis" (μ = 0.33 d^{-1}), "*Ca.* Brocadia sinica", and "*Ca.* Scalindua sp.".[17,35,36] Our semi-CSTR was operating for more than three months under a steady dilution rate of ~0.33 d^{-1}, indicating a specific growth rate (μ) of at least 0.33 d^{-1} for "*Ca.* Kuenenia stuttgartiensis", which is higher than the previously reported value for this species.[17] The higher specific growth rate reported here is advantageous because it allows higher dilution and nitrogen loading rates, thereby enabling a more efficient and stable anammox process. Because we have not done a detailed analysis of the effect of the different cultivation parameters used with our reactor, we cannot unambiguously connect any one parameter with this high growth rate. Compared to membrane bioreactors, the basic design of our semi-CSTR is simpler and does not

require a sludge pump, a gas purging system, a water level sensor, or a membrane unit. Omission of the membrane unit is especially advantageous as the membrane contributes significantly to the investment and maintenance costs of membrane bioreactors, as the membrane unit needs to be periodically cleaned after clogging or formation of biofilms on the membrane fibers.[31] Our microprocessor-controlled online-monitoring and controlling system introduces some complexity into our system, however, the monitoring and controlling system is low-cost, flexible and implementable without advanced programming knowledge.

When built as a laboratory-scale reactor, the CSTR design is very useful to precisely study the microbiology, physiology and biochemistry of anammox bacteria due to its simple structure and planktonic cell yield. Moreover, without the need for a gas supply system in our semi-CSTR, it is possible to monitor headspace pressure change for rapid determination of anammox activity inside the reactor (SI, Figure S2). The CSTR design may also be used in full scale wastewater treatment in the future despite some apparent limitations. One of the limitations is that the dilution rate in CSTR cannot be set above the growth rate of the anammox organisms, otherwise they will be diluted out of the reactor. Membrane bioreactors, as well as other biomass-retaining reactors, decouple the solid retention time from the hydraulic retention time, and therefore can be run with higher dilution rates. Nevertheless, our semi-CSTR has already achieved a nitrogen removal rate of 0.21 kg-N m^{-3} d^{-1} when operated at a dilution rate of 0.33 d^{-1} and an influent nitrite concentration of 20 mM. This value is only slightly lower than the minimum nitrogen removal rate (0.26 kg-N m^{-3} d^{-1}) in existing full scale anammox plants.[37] Another limitation of CSTR is that CSTR reactors are not able to provide biomass-free effluent and therefore are more suitable in a side-stream process where the reactor effluent can be directed to the mainstream process. With the above considerations, we believe the CSTR design has its place in treating high-ammonium-content wastewater as a side-stream process and would be especially attractive when a simple reactor design and low reactor maintenance are desired.

337 **Influence of oxygen and the redox potential on anammox**

338 Although anammox organisms can survive phases of oxic treatment in the lab, anammox activity is
339 extremely sensitive to oxygen.[29] On the other hand, anammox organisms are dependent on the
340 presence of nitrite, which is not available under strictly anoxic conditions. Therefore, anammox
341 bacteria are typical redox gradient organisms both in freshwater and marine habitats where they thrive
342 in so-called "oxygen minimum zones".[38,39] In a membrane bioreactor system, continuous bubbling
343 with 95% argon and 5% CO_2 was needed to maintain an oxygen-free environment.[31,40] In contrast to
344 this intensive and expensive gas sparging, our CSTR design using degassed feeding solutions did not
345 require continuous gas purging to obtain stable growth of anammox cells.

346 In previous experiments oxygen also had a striking effect on the growth form of Planktomycetes: cells
347 of "*Ca.* Brocadia sapporoensis" started to aggregate in a membrane bioreactor when the oxygen
348 loading rate was > 0.68 μmol-O_2 L^{-1} d^{-1}.[40] In our semi-CSTR, cells of "*Ca.* Kuenenia stuttgartiensis"
349 were in their planktonic form throughout all reactor operations (Figure 4 and SI, Figure S3). Even after
350 exposure to air (21% oxygen) for 2 hours, no aggregates were formed in the reactor effluent as
351 observed by epifluorescence microscopy (images not shown). In summary, "*Ca.* Brocadia
352 sapporoensis" seems to aggregate more easily upon oxygen exposure than "*Ca.* Kuenenia
353 stuttgartiensis".

354 Apart from dissolved oxygen, the redox potential is a parameter that greatly influences anammox
355 activities. Previous studies showed that anammox activities were maximal around 0 mV (vs SHE).[28,41]
356 Indeed, the redox potential within our semi-CSTR stabilized at around 10 mV (± 15 mV) (vs SHE) as
357 long as the reactor was in operation (SI, Figure S2). The self-stabilization of the redox potential
358 without external manipulation such as reductant addition or gas purging is an interesting phenomenon,
359 and the underlying mechanism is worth further investigation. Currently we cannot attribute this redox
360 stabilization activity to either the anammox catalyzing population in the reactor or accompanying
361 populations.

Growth rate and growth yield

Since the demonstration of relatively fast growth of anammox bacteria in membrane bioreactors anammox organisms have no longer been considered as slow-growing microorganisms.[36] So far, the highest reported maximum growth rates for anammox bacteria were 0.33 d^{-1} for "*Ca.* Brocadia"[15,36] and 0.18 d^{-1} for "*Ca.* Jettenia",[15] corresponding to a steady state solid retention time of 3 days and 5.6 days, respectively. With our reactor design we gradually decreased the hydraulic retention time to 3 days within 16 days of reactor operation, corresponding to a dilution rate and bacterial specific growth rate of μ = ~0.33 d^{-1} at the steady state (SI, Figure S1). This was achieved without intensive optimization of temperature, pH, or the medium recipe, and therefore there might be room for improvement of the growth rate. For example, a relatively low iron concentration of 15 μM was used in this study while a higher iron concentration may be beneficial.[36,42] Also, adjusting the phosphate concentration could enhance anammox activity as shown in the batch test (Figure 5D).

When the culture exhibited the highest growth rate, the specific anammox activity was 3.03 g NH_4 g^{-1} VSS d^{-1}. This value is close to the one reported for "*Ca.* Brocadia sapporoensis" (3.38 g NH_4 g^{-1} VSS d^{-1}).[36] Given that these two species exhibited similar maximum growth rates and specific anammox activity, these values may have approached the limit for freshwater anammox cells.

Anammox stoichiometry

The anammox stoichiometry for "*Ca.* Kuenenia stuttgartiensis" in our reactor was determined as given in Equation 1 based on a two-week reactor operation and we compared this equation to two previously determined equations for "*Ca.* Brocadia anammoxidans" (Equation 2)[33] and "*Ca.* Brocadia sapporoensis" (Equation 3)[40] (Table 1). Our calculations are based on the two different biomass formulae described by Strous *et al.* (1998) and Lotti *et al.* (2014), respectively. Two important values in this calculation are the moles of consumed nitrite per mole of consumed ammonium (NO_2/NH_4) and the moles of produced nitrate per mole of consumed ammonium (NO_3/NH_4). In such a comparison our NO_2/NH_4 ratios of 1.270 and 1.249 for the two biomass formulae assumptions, respectively, are between the NO_2/NH_4 ratio obtained by Strous *et al.* (1998) (1.320) and that obtained by Lotti *et al.*

(2014) (1.146). The NO_3/NH_4 ratio in our reactor (0.238 and 0.239 for the two biomass formula, respectively) is similar to that calculated by Strous *et al.* (1998) (0.258) but higher than the ratio calculated by Lotti *et al.* (2014) (0.161). Such a difference could be due to specific properties of the strains used. However, even the NO_3/NH_4 ratios for the two *Brocadia* strains differ strongly from each other. The NO_3/NH_4 ratio of 0.161 given by Lotti *et al.* (2014) contradicts their own stable measurements (Figure 5D in Lotti *et al.* (2014)) of 0.2 ± 0.01 for molar NO_2/NH_4 ratios and also contradicts the ratio of 0.210 obtained by us after recalculation and reconciliation of their original data (Supporting Table 2A in Lotti *et al.* (2014)) without constraints on the N-source (Table 1). This, the almost perfectly closed mass balance in our measurements and the very low errors between observed and calculated coefficients for our reactor support the precision of our formula. The cultivation in a homogeneous system with a highly enriched anammox culture contributes to this precision.

The biomass yield in our study with "*Ca.* Kuenenia stuttgartiensis" (1.97 g VSS per mole consumed ammonium) was higher than with the two "*Ca.* Brocadia" species (1.70 and 1.57, respectively), possibly due to intrinsic properties of the used "*Ca.* Kuenenia" species in comparison with "*Ca.* Brocadia" species.

$$1\,NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ = 1.02\,N_2 + 0.26\,NO_3^- + 0.066\,CH_2O_{0.5}N_{0.15} + 2.03\,H_2O$$ Equation 2, Strous *et al.* (1998)

$$1\,NH_4^+ + 1.146\,NO_2^- + 0.071\,HCO_3^- + 0.057\,H^+ = 0.986\,N_2 + 0.161\,NO_3^- + 0.071\,CH_{1.74}O_{0.31}N_{0.2} + 2.002\,H_2O$$ Equation 3, Lotti *et al.* (2014)

Microbial population changes during enrichment

Bacteria of the genus *Pseudomonas*, which accounted for 70.3% of the total detected 16S rRNA genes in the batch cultures used as reactor inoculum, almost disappeared after three-months of reactor operation. This relative high abundance of *Pseudomonas* species in the batch cultures might be due to denitrifying activity making use of organic components as electron donors and nitrate/nitrite as electron acceptors. *Pseudomonas* species have previously been described as denitrifying bacteria, for example *Pseudomonas xiamenensis*.[43] *Pseudomonas bauzanensis* was described as a non-denitrifying bacterium,[44] but its genome (NCBI accession number JFHS00000000.1) contains the full set of denitrification genes suggesting that its denitrification activity might have not yet been discovered.

The disappearance of potentially denitrifying cells of the genus *Pseudomonas* in the reactor over time might therefore be due to the fact that the dilution rate of the reactor exceeded their maximum growth rate under reactor conditions and a competition for electron donors with other heterotrophs.

Several heterotrophs emerged during reactor operation, including *Methyloversatilis* which accounted for more than 10% of the total detected 16S rRNA genes in the reactor after 3 months. *Methyloversatilis* strains are facultative methylotrophs growing on a variety of organic compounds.[45] *Chloroflexi* were often reported to co-exist with anammox bacteria and scavenge organic compounds derived from anammox bacterial cells.[46] However, in reactors with fast dilution rates and active anammox populations, decayed anammox cells should be minimal. Indeed, in our reactor, *Chloroflexi* accounted for only 0.019% of the total detected 16S rRNA genes.

Anammox abundance in our semi-CSTR reached around 87% as shown by Illumina amplicon sequencing and FISH, lower than what membrane bioreactor systems usually achieved (>95%).[31] This is probably because the semi-CSTR was fed with only 20 mM nitrite while membrane bioreactor influent typically contained 60 mM nitrite. A higher nitrite concentration can lead to higher cell numbers of anammox organisms in the reactor whereas heterotrophs in the reactor are limited by trace amounts of organics in the influent. The abundance of anammox bacteria is expected to increase when influent nitrite is elevated and when the hydraulic retention time is further reduced but this was not the goal in the current study.

Effluent incubations

The monitored effects in our incubations of reactor effluent with inhibitors are due to the combined inhibitory effects on anammox enzymatic activity and on anammox cell growth. However, because the incubation time was relatively short and only 3 mM nitrite was added (except for tests of nitrite effects) compared to 20 mM nitrite in the reactor influent, cell growth during the experiment should be low.

Our results that salt concentrations of 120-270 mM start to inhibit anammox activity is consistent with previous findings suggesting a salt tolerance range of between 50 and 200 mM.[3] Slightly higher sensitivity for nitrite and sulfide was found in the reactor effluent compared to the literature.[3] While

we found that nitrite caused severe inhibition at 10 mM, previous reports described "*Ca.* Kuenenia stuttgartiensis" to be tolerant to 13-25 mM nitrite.[47,48] The observed inhibition by sulfide in our experiments was even stronger than those reported with planktonic cells from membrane bioreactors,[49,50] and was orders of magnitude lower than values obtained with cell aggregates.[51] "*Ca.* Kuenenia stuttgartiensis" were found to be relatively insensitive to chloramphenicol when dispersed anammox granules were used for anammox activity tests, with IC_{50} values of ~400 mg L^{-1} [52] or even >1 g L^{-1} [48]. Interestingly, the study that observed an IC_{50} of ~400 mg L^{-1} on dispersed anammox granules also showed that as low as 20 mg L^{-1} chloramphenicol caused a 25% drop in reactor efficiency and a 75% drop in specific anammox activity after 4 days incubation in the same reactor that provided anammox granules for activity tests.[52] It is therefore possible that in these two studies the relatively short incubation time of ~6 h in anammox activity tests was not sufficient to fully reveal the inhibition effects by chloramphenicol, and the inhibition effects became prominent only after prolonged incubation (>4 d) in the reactor. In our study, 30 mg L^{-1} chloramphenicol caused 57% inhibition within 10.4 h of incubation (SI, Figure S5). These results suggest that the planktonic cells in our study were more readily accessible for the effector and inhibition was observable within a shorter incubation time. However, we cannot exclude that the previously investigated "*Ca.* Kuenenia" strains display undescribed antibiotic resistance.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: …

Population composition of the initial anammox culture (Table S1); data-set used for stoichiometry calculation (Table S2); hydraulic retention time in the reactor during start-up (Figure S1); gas pressure and redox changes over time in the reactor after discontinuing medium amendments (Figure S2); epifluorescence microscopic image of the SYBR Green-stained reactor culture (Figure S3); influencing parameters on cell counting by epifluorescence

467 microscopy (Figure S4); gas production in cultures with reactor effluent amended with different
468 chloramphenicol concentrations (Figure S5); supporting methods

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475 **Notes**

476 The authors declare no competing financial interest.

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Tables

Table 1: Comparison of anammox stoichiometry coefficients for "*Ca.* Kuenenia" and two "*Ca.* Brocadia" species.

	Coefficient in stoichiometric equations											
Species	"*Ca.* Kuenenia stuttgartiensis"				"*Ca.* Brocadia anammoxidans"		"*Ca.* Brocadia sapporoensis"					
Reference of input data	This study				Strous *et al.* (1998) [33]		Lotti *et al.* (2014) [40]					
Biomass formula	$CH_2O_{0.5}N_{0.15}S_{0.05}$		$CH_{1.74}O_{0.31}N_{0.2}S_{0.01}P_{0.01}$		$CH_2O_{0.5}N_{0.15}S_{0.05}$		$CH_{1.74}O_{0.31}N_{0.2}S_{0.01}P_{0.01}$					
Constraint	No N-source constraints						NH_4 as N-source		No N-source constraints			
Measured biomass yield [a]	-						0.071 C-mol NH_4-mol^{-1}				0.059 C-mol NH_4-mol^{-1}	
Compound	Value	Diff [b] %	Value	Diff %	Value	Diff %	Value	Diff %	Value	Diff %	Value	Diff %
NH_4^+	1	-0.4	1	1.1	1	-5.5	1	6.6	1	1.0	1	-1.4
NO_2^-	1.270	-0.1	1.249	-0.3	1.320	3.0	1.146	0.7	1.225	0.0	1.250	1.6
HCO_3^-	0.077	-	0.087	-	0.066	-3.7	0.071	-	0.073		0.061	-
H^+	0.108	-	0.097	-	0.128	-	0.057	-	0.088 [c]		0.099	-
N_2	1.010	0.3	0.996	0.4	1.026	-0.9	0.986	-	1.000		1.013	-
NO_3^-	0.238	0.6	0.239	2.2	0.258	-4.1	0.161	-17.9	0.210	0.4	0.212	-0.0
Biomass	0.077	-0.4	0.087	-3.4	0.066	5.0	0.071	6.6	0.073	3.2	0.061	0.9
H_2O	2.016	-	2.016	-	2.031	-	2.002	-	1.950	-	2.027	-
Yield [d]	1.97		1.93		1.70		1.57		1.62		1.34	

[a] Lotti *et al.* (2014) gave conflicting biomass yield values (in C-mol NH_4-mol^{-1}) of 0.071 as given directly in their text and 0.059 as calculated from data in Table 2A in their supporting online material.

[b] "Diff": difference in percent between the measured conversion rates and the calculated balanced conversion rates according to the corresponding coefficients.

[c] The coefficient for proton 0.024 as given in Table 3 by Lotti *et al.* (2014) caused imbalance in charge and element. Here we give the inferred value of 0.088 based on charge and element balance.

[d] Biomass yield calculated based on the stoichiometry, unit: g VSS per mole of consumed ammonium.

Figures

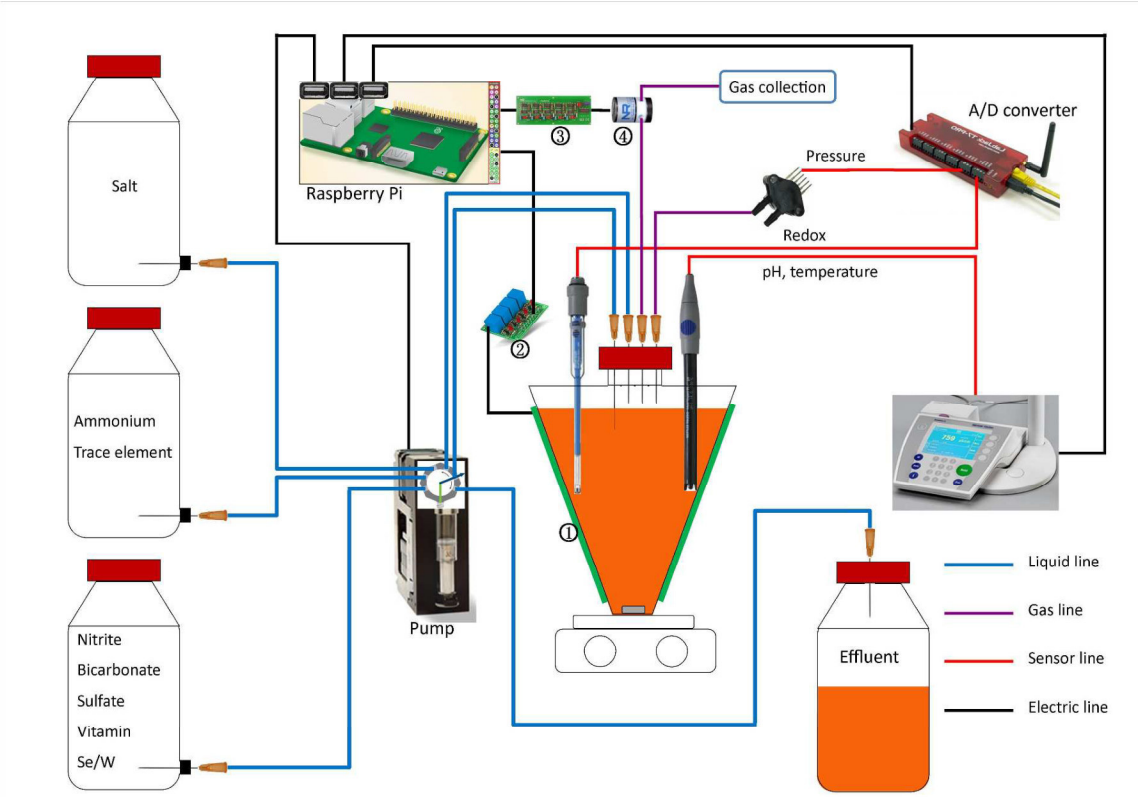
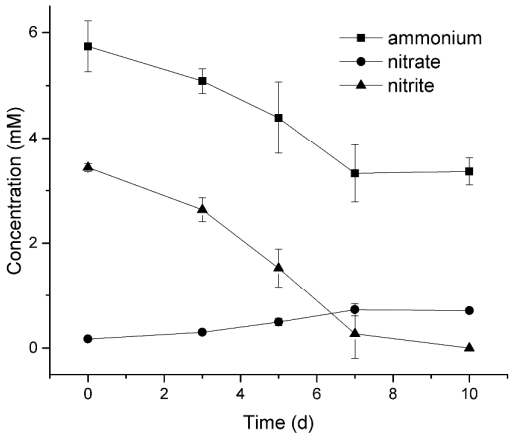
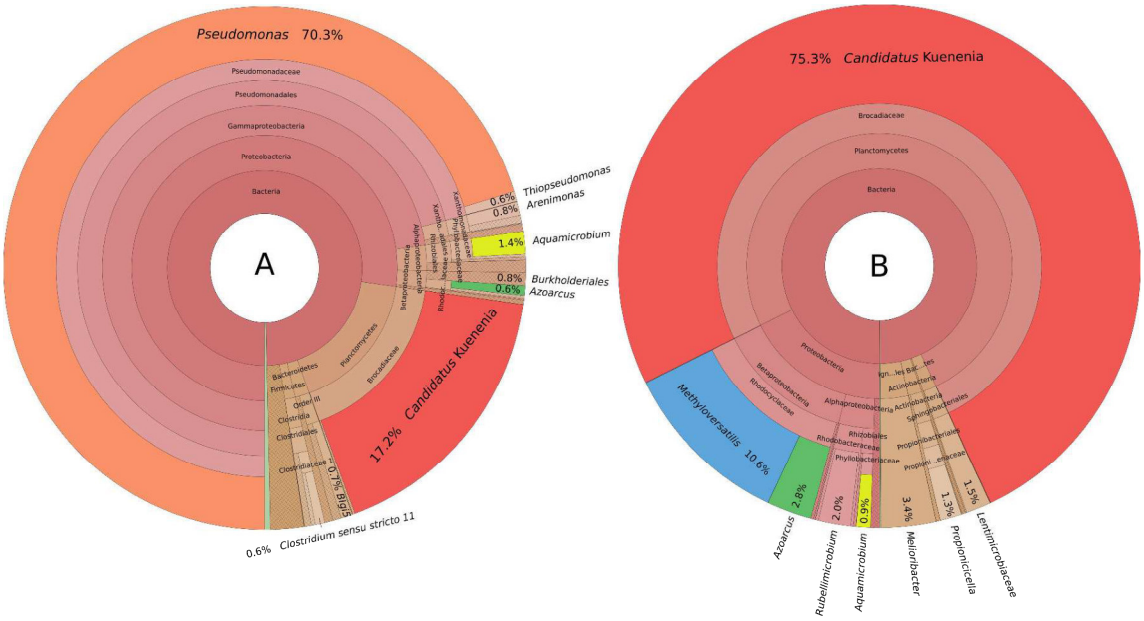


Figure 1: Schematic layout of the semi-continuous stirred-tank reactor. Labels: (1) Heating blanket, (2) Relay for heating blanket, (3) Relay for gas valve, (4) Gas valve. All medium bottles and the effluent bottle were pressurized with a gas mixture of 80% N_2 and 20% CO_2 at 10 kPa (not shown in the figure).



640

641 Figure 2: Anammox activity in a sludge- and granule-free planktonic batch cultures after three
642 transfers. Shown are means ± SD of triplicate cultures. Nitrogen evolution and cell growth were not
643 measured here.



644

645 Figure 3: Illumina amplicon sequencing analyses of the microbial communities (A) in the granule-free
646 batch culture after three transfers and (B) in the reactor after three months of operation. Common and
647 unique abundant (>10%) populations are labeled. Shown are data based on the absolute numbers of
648 detected 16S rRNA genes not taking into account different copy numbers of 16S rRNA genes per
649 genome in the respective populations.

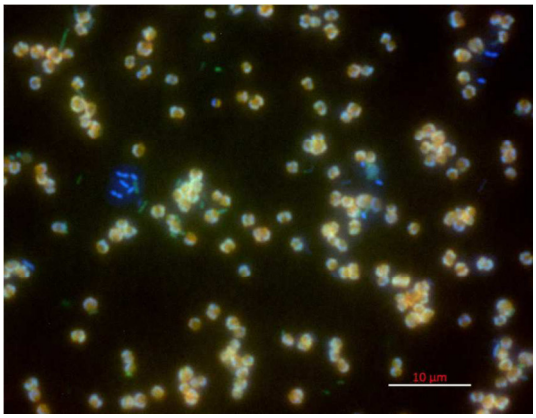
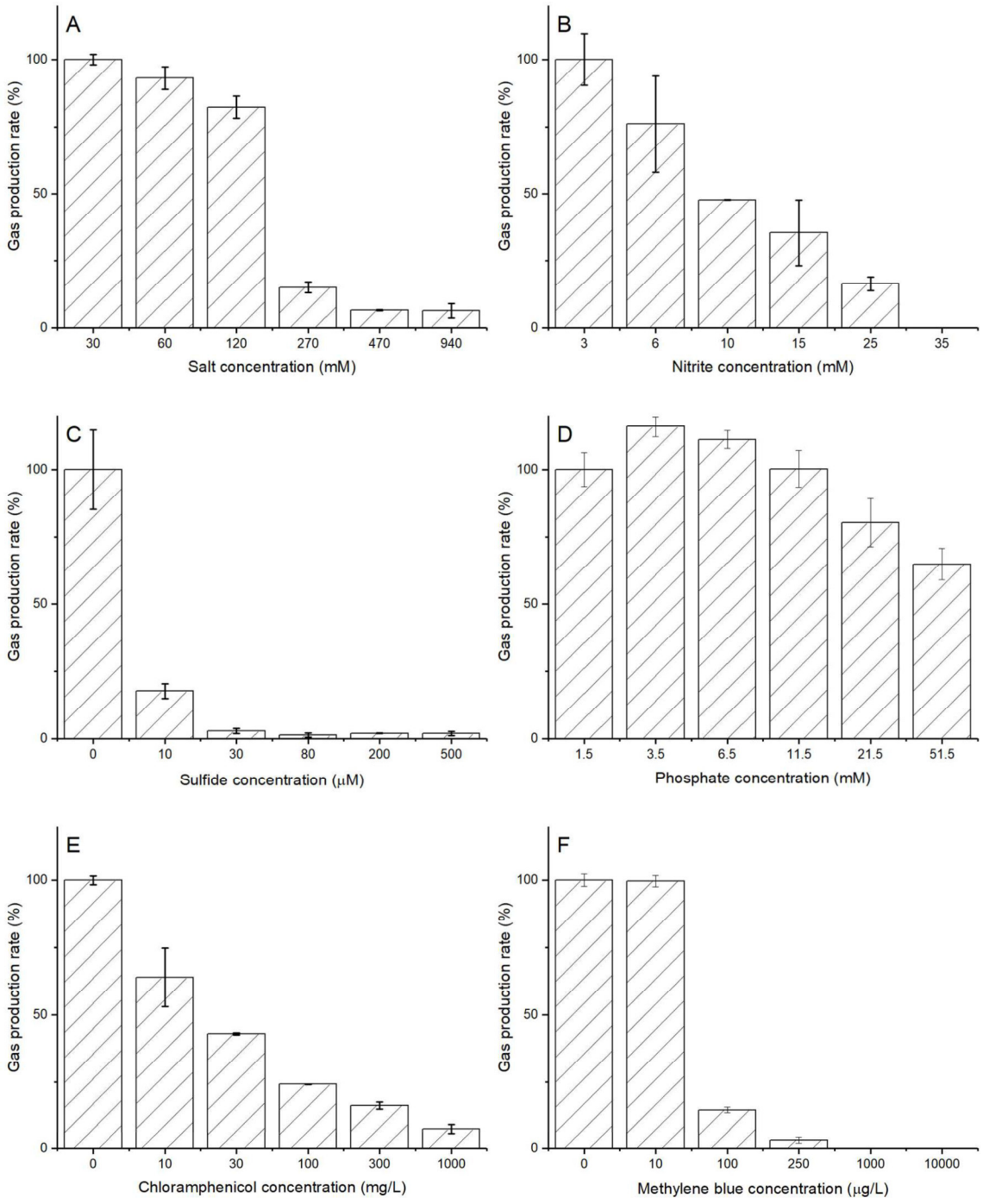


Figure 4: Fluorescence *in situ* hybridization of the reactor culture with FAM-labeled Eub338-mix (green), cy5-labeled AMX820 (red) and counter stained with DAPI (blue). Anammox cells are light blue on the edges due to DAPI-stained high DNA content in the cytoplasm ('riboplasm' in Planctomyetes); the center of the anammox organisms ('anammoxosome') is DNA-free and stains orange/brown due to three overlapping weak signals from the two FISH probes and DAPI from the over/underlaying riboplasm. Cells that do not hybridize with AMX820 are blue or green.



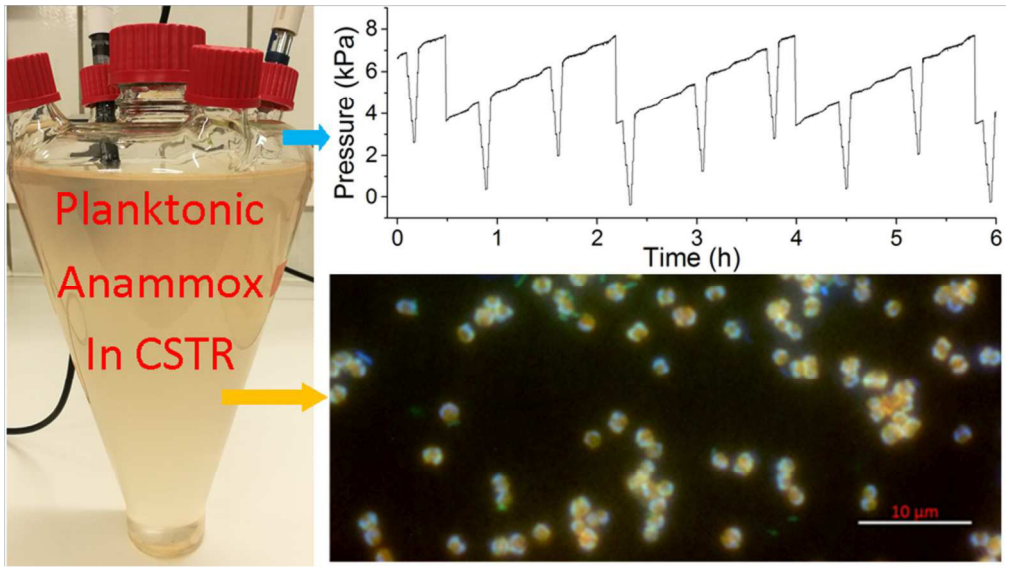
657

658 Figure 5: Influence of different effectors on anammox activity of reactor effluent. (A) Salt (NaCl); (B)

659 Nitrite ($NaNO_2$); (C) Sulfide (Na_2S); (D) Phosphate (mixture of K_2HPO_4 and KH_2PO_4, pH 7.2); (E)

660 Chloramphenicol; (F) Methylene blue. Gas production rates were normalized against positive controls

661 with no effectors and 3 mM nitrite which produced 70-120 mL N_2 L^{-1} d^{-1} at 30°C.



TOC-Graphic

84x47mm (300 x 300 DPI)